**Additional file 2: Additional tables**

**CRISPR-Cas9-mediated genomic multiloci integration in** ***Pichia pastoris***

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# Table S1 Gene integration efficiencies in *P. pastoris* Δ*ku70* strain.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Type of integration** | **Liquid Medium** | **Locus\_reporter protein** | **CFUs/**  **μg DNAa** | **Correct/**  **analyzed colonies** | **Integration efficiency (%)** |
| Single-locus | YND | P*TEF1*UP-g1\_eGFP | 3296 | 92/96 | 95.8 |
| P*TEF1*UP-g2\_eGFP | 1072 | 91/96 | 94.8 |
| P*GAP*UP-g1\_eGFP | 4480 | 66/88 | 76.1 |
| P*GAP*UP-g2\_eGFP | 1250 | 84/95 | 88.4 |
| P*FLD1*UP-g1\_eGFP | 462 | 88/96 | 91.7 |
| P*FLD1*UP-g2\_eGFP | 110 | 50/55 | 90.9 |
| P*AOX1*UP-g1\_eGFP | 2800 | 92/93 | 98.9 |
| P*AOX1*UP-g2\_eGFP | 2765 | 90/95 | 94.7 |
| P*AOX1*UP-g3\_eGFP | 2260 | 91/96 | 94.8 |
| *AOXTT*DOWN\_eGFP | 725 | 60/93 | 64.5 |
| YNDH | P*TEF1*UP-g1\_eGFP | 3296 | 94/96 | 97.9 |
| P*GAP*UP-g2\_eGFP | 1250 | 72/96 | 75.0 |
| P*FLD1*UP-g1\_eGFP | 462 | 90/96 | 93.8 |
| P*AOX1*UP-g2\_eGFP | 2765 | 86/96 | 89.6 |
| *AOXTT*DOWN\_eGFP | 725 | 72/96 | 75.0 |
| Double-locus | YNDH | P*TEF1*UP-g1\_eGFP  P*AOX1*UP-g2\_mCherry | 107 | 31/48 | 64.6 |
| P*AOX1*UP-g2\_eGFP  P*TEF1*UP-g1\_ mCherry | 76 | 30/48 | 62.5 |
| P*TEF1*UP-g1\_eGFP  P*FLD1*UP-g1\_ mCherry | 26 | 15/26 | 57.7 |
| P*FLD1*UP-g1\_eGFP  P*TEF1*UP-g1\_ mCherry | 48 | 14/20 | 70.0 |
| Triple-locus | YNDH | P*TEF1*UP-g1\_eGFP  P*AOX1*UP-g2\_mCherry  P*FLD1*UP-g1\_BFP | 30 | 6/30 | 20.0 |
| P*TEF1*UP-g1\_eGFP  P*FLD1*UP-g1\_mCherry  P*AOX1*UP-g2\_BFP | 28 | 9/28 | 32.1 |
| P*AOX1*UP-g2\_eGFP  P*TEF1*UP-g1\_ mCherry  P*FLD1*UP-g1\_BFP | 24 | 3/24 | 12.5 |
| P*AOX1*UP-g2\_eGFP  P*FLD1*UP-g1\_ mCherry  P*TEF1*UP-g1\_BFP | 28 | 5/28 | 17.9 |
| P*FLD1*UP-g1\_eGFP  P*TEF1*UP-g1\_ mCherry  P*AOX1*UP-g2\_BFP | 39 | 9/39 | 23.1 |
| P*FLD1*UP-g1\_eGFP  P*AOX1*UP-g2\_ mCherry  P*TEF1*UP-g1\_BFP | 20 | 4/20 | 20.0 |
| Multiple-copy | YNDH | P*AOX1*UP-g2\_eGFP  P*TEF1*UP-g1\_eGFP | 102 | 19/24 | 79.2 |
| P*FLD1*UP-g1\_eGFP  P*TEF1*UP-g1\_ mCherry  P*AOX1*UP-g2\_BFP | 39 | 9/39 | 23.1 |

a The CFUs were calculated based on the number of colonies that grew on the YND plate after transformation. Then colonies were picked and transferred into liquid medium (YND or YNDH) for analysis of integration analysis.

# Table S2 Primers used in this study

|  |  |
| --- | --- |
| **Primer** | **Sequence (5’→3’)** |
| **Primers for construction of pPIC3.5K-KU70-gRNA1** | |
| PARS-F | CACAAGGGTCTCGAGATAAGCTGGGGGAAC |
| PARS-R | CACCTGACGTTCGACAATTAATATTTACTTATTTTGGTCAAC |
| DAS1TT-R-2 | CTTATCTCGAGACCCTTGTGACTGACACTT |
| DAS1TT-F | GAAGGTGTGAACGGGAAGTCTTTACAGTTT |
| HTX1-R | TCAGTTTGATTTTGATTTGTTTAGGTAACT |
| HTX1-F | TCTTGTCCATCGTTTCGTGTTGTAGTTTTAATATAGT |
| Cas9(NLS)-R | GACTTCCCGTTCACACCTTCCTCTTCTTCT |
| Cas9(NLS)-F | AACTACAACACGAAACGATGGACAAGAAGTACTCCAT |
| pHTX1-HH-F | AGTTACCTAAACAAATCAAAAAGATGCTGATGAGTCCGTGAGG |
| Plasmid-PARS | TAATTGTCGAACGTCAGGTGGCACTTTTC |
| 3AOX1F | GGATGTCAGAATGCCATTTG |
| 3AOX1 | CAAATGGCATTCTGACATCC |
| **Primers for construction of gRNA-Cas9 plasmids** | |
| inOri R | GGGAGAAAGGCGGACAGGTA |
| inOri F | TACCTGTCCGCCTTTCTCCC |
| KU70-gRNA2-F | ACAAATCAAAGCAAGGCTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCCCTTGCCCGGTACAGGAATAGTTTTAGAGCTAGAAATAGCAAGT |
| gRNA2-KU70-HTX-R | TCAGCCTTGCTTTGATTTGTTTAGGTAACTTGAAC |
| GUT1-gRNA1-F | ACAAATCAAATACTCGCTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCCGAGTACTCTACCTCTGCTCGTTTTAGAGCTAGAAATAGCAAGT |
| gRNA1-HTX-R | TCAGCGAGTATTTGATTTGTTTAGGTAACTTGAAC |
| GUT1-gRNA2-F | ACAAATCAAAATTGCACTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCTGCAATTTCCTCAGCCAGGCGTTTTAGAGCTAGAAATAGCAAGT |
| gRNA2-HTX-R | TCAGTGCAATTTTGATTTGTTTAGGTAACTTGAAC |
| GUT1-gRNA3-F | ACAAATCAAAAACAACCTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCGTTGTTTGGTCCAAGAAGACGTTTTAGAGCTAGAAATAGCAAGT |
| gRNA3-HTX-R | TCAGGTTGTTTTTGATTTGTTTAGGTAACTTGAAC |
| PTEF1-gRNA1-F | ACAAATCAAATCTTGCCTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCGCAAGATGGTTAAAAGGTGAGTTTTAGAGCTAGAAATAGCAAGT |
| PTEF1-gRNA1-HTX-R | CTCATCAGGCAAGATTTGATTTGTTTAGGTAACTTGAACTGG |
| PTEF1-gRNA2-F | ACAAATCAAACCATTCCTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCGAATGGGCAAGATGGTTAAAGTTTTAGAGCTAGAAATAGCAAGT |
| PTEF1-gRNA2-HTX-R | CTCATCAGGAATGGTTTGATTTGTTTAGGTAACTTGAACTGG |
| PGAP-gRNA1-F | ACAAATCAAAATCGATCTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCATCGATAATAGTCGCATGTGGTTTTAGAGCTAGAAATAGCAAGT |
| PGAP-gRNA1-HTX-R | TCAGATCGATTTTGATTTGTTTAGGTAACTTGAACTGG |
| PGAP-gRNA2-F | ACAAATCAAATTAAAACTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCTTTTAAGATTTCAATCTTGAGTTTTAGAGCTAGAAATAGCAAGT |
| PGAP-gRNA2-HTX-R | TCAGTTTTAATTTGATTTGTTTAGGTAACTTGAACTGG |
| AOXTT-gRNA1-F | ACAAATCAAAGCGTCACTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCTGACGCTTATTATACCCTTTGTTTTAGAGCTAGAAATAGCAAGT |
| AOXTT-gRNA1-HTX-R | TCAGTGACGCTTTGATTTGTTTAGGTAACTTGAACTGG |
| PFLD1-gRNA1-F | ACAAATCAAATGCCGCCTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCGCGGCAGTAATTGATATCGTGTTTTAGAGCTAGAAATAGCAAGT |
| PFLD1-gRNA1-HTX-R | CTCATCAGGCGGCATTTGATTTGTTTAGGTAACTTGAACTGG |
| PFLD1-gRNA2-F | ACAAATCAAAATTACTCTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCAGTAATTGATATCGTAGGGTGTTTTAGAGCTAGAAATAGCAAGT |
| PFLD1-gRNA2-HTX-R | CTCATCAGAGTAATTTTGATTTGTTTAGGTAACTTGAACTGG |
| PAOX1-gRNA1-F | ACAAATCAAATGGATTCTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCAATCCAAATGTCATCATTGTGTTTTAGAGCTAGAAATAGCAAGT |
| PAOX1-gRNA1-HTX-R | TCAGAATCCATTTGATTTGTTTAGGTAACTTGAACTGG |
| PAOX1-gRNA2-F | ACAAATCAAAAGGCGCCTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCGCGCCTACAATGATGACATTGTTTTAGAGCTAGAAATAGCAAGT |
| PAOX1-gRNA2-HTX-R | TCAGGCGCCTTTTGATTTGTTTAGGTAACTTGAACTGG |
| PAOX1-gRNA3-F | ACAAATCAAAAATCCACTGATGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCTGGATTTGGTTGACTCATGTGTTTTAGAGCTAGAAATAGCAAGT |
| PAOX1-gRNA3-HTX-R | TCAGTGGATTTTTGATTTGTTTAGGTAACTTGAACTGG |
| **Primers for construction of donor cassette plasmids bearing reporter gene** | |
| Hind-Kdown-R | GTAAAACGACGGCCAGTGCCAAGCTTCAATACCGATAAAGTGGTCAACT |
| MSC-Kdown-F | CGAGCTCACTAGTGGTACCGTGTTCCTTACTTTTTCCTCGCA |
| MCS-Kup-R | CGGTACCACTAGTGAGCTCGGCTAAGTGTGAGAAGAAGAGA |
| EcoR-Kup- F | ACAGCTATGACCATGATTACGAATTCCACGGGTGATTACTTGTTTACAT |
| MCS-Gup-R | CGGTACCACTAGTGAGCTCGTAGAAGAAGAGTCTTTTTCAGTCC |
| EcoR-Gup- F | ACAGCTATGACCATGATTACGAATTCAAATCTAGGTCATCCTACAGCAAA |
| Hind-Gdown-R | GTAAAACGACGGCCAGTGCCAAGCTTAACGTTCGTATCGTGATCTTG |
| GFP-F | ATGATTACGAATTCGAGCTCTGACGCTCAGTGGAACGAAA |
| GFP-R | CTAGAGGATCCCCGGGTACCTTGAAGCTATGGTGTGTGGGG |
| MSC-Gdown-F | CGAGCTCACTAGTGGTACCGAGCAGCTGTAATTATATTATCATGTTAGG |
| OL2-Xba1-DO-F | GGTACCCGGGGATCCTCTAGA |
| OL2-Sal1-UP-R | TTGCATGCCTGCAGGTCGAC |
| HA-TEFg1DO-F | GGTACCCGGGGATCCTCTAGACCTTTTAACCATCTTGCCCATTC |
| HA-TEFg1DO-R | GGGCCCCTCGAGACTAGTGGGTTGTAACCAACCTTCTTGAT |
| HA-PTEFUP-F | ACTAGTCTCGAGGGGCCCGTGGACTTTCTTAGGAGAGTCACTA |
| HA-TEFg1UP-R | TTGCATGCCTGCAGGTCGACTGAAGGAGGCCAGACAGGATT |
| HA-TEFg2DO-F | GGTACCCGGGGATCCTCTAGAAACCATCTTGCCCATTCCAACT |
| HA-TEFg2UP-R | TTGCATGCCTGCAGGTCGACAAAAGGTGAAGGAGGCCAGAC |
| HA-pAOX1-DOWN-R | GGGCCCCTCGAGACTAGTGGGATAGCCATCGTTTCGAATAA |
| HA-PAOX1-UP-F | ACTAGTCTCGAGGGGCCCCTGAGAGTACATCGGTTTCAAAAGG |
| HA-pAOX1-g1-DOWN-F | GGTACCCGGGGATCCTCTAGAATGATGACATTTGGATTTGGTTGAC |
| HA-pAOX1-g1-UP-R | TTGCATGCCTGCAGGTCGACTGTAGGCGCTGGGATTTCAGG |
| HA-pAOX1-g2-DOWN-F | GGTACCCGGGGATCCTCTAGAATTTGGATTTGGTTGACTCATGTTGG |
| HA-PAOX1-g2-UP-R | TTGCATGCCTGCAGGTCGACGTCATCATTGTAGGCGCTGGGAT |
| HA-pAOX1-g3-DOWN-F | GGTACCCGGGGATCCTCTAGATGTTGGTATTGTGAAATAGACGCA |
| HA-pAOX1-g3-UP-R | TTGCATGCCTGCAGGTCGACTGAGTCAACCAAATCCAAATGTC |
| HA-AOXTT-g1-DOWN-R | GGGCCCCTCGAGACTAGTGACTCGTGTGTTGGCCAGTAA |
| HA-AOXTT-g1-UP-F | ACTAGTCTCGAGGGGCCCTCCAGAGGTTCCATTCACATTAC |
| HA-AOXTT-g1-DOWN-F | GGTACCCGGGGATCCTCTAGAGGGTATAATAAGCGTCATTTGCAGC |
| HA-AOXTT-g1-UP-R | TTGCATGCCTGCAGGTCGACTTTTGGCATCGTTGAAGCTTGCA |
| HA-pGAP-DOWN-R | GGGCCCCTCGAGACTAGTGGAGCCAAACAGTTGGTAGTAC |
| HA-PGAP-UP-F | ACTAGTCTCGAGGGGCCCAAAACTGGTCTGCCAAGCACA |
| HA-PGAP-g1-DOWN-F | GGTACCCGGGGATCCTCTAGAATGCGACTATTATCGATCAATGAAATCC |
| HA-PGAP-g1-UP-R | TTGCATGCCTGCAGGTCGACGTGAGGCTGAAATGTGCCGA |
| HA-PGAP-g2-DOWN-F | GGTACCCGGGGATCCTCTAGAAGATTGAAATCTTAAAATTGCCCC |
| HA-PGAP-g2-UP-R | TTGCATGCCTGCAGGTCGACTGATGGATTTCATTGATCGAT |
| HA-PFLD1UP-F | ACTAGTCTCGAGGGGCCCGCCCAATCTGTTGTCCCCAAACATAA |
| HA-PFLD1DO-R | GGGCCCCTCGAGACTAGTCTACAGAATCCCCAACCTTCACG |
| HA-PFLD1g1UP-R | TTGCATGCCTGCAGGTCGACATATCAATTACTGCCGCATTGG |
| HA-PFLD1g1DO-F | GGTACCCGGGGATCCTCTAGACGTAGGGTAGGTCTGGAAAGA |
| HA-PFLD1g2DO-F | GGTACCCGGGGATCCTCTAGAGGTAGGTCTGGAAAGACGCT |
| HA-PFLD1g2UP-R | TTGCATGCCTGCAGGTCGACCTACGATATCAATTACTGCCGCA |
| pGAPDO-R | CCATGGTCCTCGTTTCGAAA |
| mChy-F | TTTCGAAACGAGGACCATGGTGAGCAAGGGC |
| pGAP R | GACGAGGACACCAAGACATT |
| pGAP F | AATGTCTTGGTGTCCTCGTC |
| **Primers for detection of gene deletion** | |
| inKU70UP-F | CTGGCCGTACACATTTCAGA |
| inKU70DO-R | GCGGAGTCTCGTTATTCATAG |
| inCas9R1 | CCGAGTGACAGGGCGATAAGA |
| gCF-GS-F | CCAGACTTTTTCTTCCCGAAT |
| inGUT1DO-R-1 | CAGTAACAGACAAATATAGTCGG |
| **Primers for construction of gRNAs-Cas9 plasmids bearing multiple RGRs** | |
| HDV-2-R | TAATTCGCGGCCGTCCCATTC |
| HDV-HH-T1-F | GAATGGGACGGCCGCGAATTATCTTGCCTGATGAGTCCGTG |
| HDVTT-R | GTCCCATTCGCCATGCCGAA |
| HDV-pHTX-HH-F | TTCGGCATGGCGAATGGGACATCAAAAGGCGCCTGATGA |
| HDV-R | GTCCCATTCGCCATGCCGAA |
| HDV-SpeI-PGg2HH-F | TTCGGCATGGCGAATGGGACACTAGTTTAAAACTGATGAGTCCGTGAGG |
| **Primers for construction of expression plasmids** | |
| TEF1UP-F | CATGATTACGAATTCGAGCTGGAAACTGTTCGTTTTTCAACTTC |
| TEF1UP-R | ACGGGCCCCTCGAGACTAGTTGAAGGAGGCCAGACAGGAT |
| TEF1DO-F | ACTAGTCTCGAGGGGCCCGTCCTTTTAACCATCTTGCCCATT |
| TEF1DO-R | TGCCTGCAGGTCGACTCTAGCTTTTGAGACCATTTGACGGAG |
| FLD1UP-F | CATGATTACGAATTCGAGCTATAACTCTTGTCGATACGTATACTTGC |
| FLD1UP-R | ACGGGCCCCTCGAGACTAGTATATCAATTACTGCCGCATTGG |
| FLD1DO-F | ACTAGTCTCGAGGGGCCCGTCGTAGGGTAGGTCTGGAAAG |
| FLD1DO-R | TGCCTGCAGGTCGACTCTAGACGGACTCAACACCTTCTCC |
| AOX1UP-F | CATGATTACGAATTCGAGCTGAGCCTCAAGGTATATAGCTATGG |
| AOX1UP-R | ACGGGCCCCTCGAGACTAGTGTCATCATTGTAGGCGCTGG |
| AOX1DO-F | ACTAGTCTCGAGGGGCCCGTATTTGGATTTGGTTGACTCATGT |
| AOX1DO-R | TGCCTGCAGGTCGACTCTAGTTTTTTGATCTTCTCAAGTTGTCG |
| HATUP-GAP-F | TCTGGCCTCCTTCAACTAGTCTCGAGCTTTTTGTAGAAATGTCTTGGTGTCC |
| HATDO-AOXTT-R | GGCAAGATGGTTAAAAGGACGGGCCCCTTCTCACTTAATCTTCTGTACTCTG |
| HAAUP-GAP-F | CCAGCGCCTACAATGATGACCACTAGTGAGCTCTTTGTGCGGATCCGAAG |
| HAADO-AOXTT-R | GTCAACCAAATCCAAATACGGGCCCTCTAGAGACATTTCTACAAAAATCTCAC |
| HAFUP-GAP-F | GCAGTAATTGATATACTAGTGGGATTTTGGTCATGCATGAG |
| HAFDO-AOXTT-R | ACCTACCCTACGACGGGCCCGGTCTCACTTAATCTTCTGTACTCTGA |
| **Primers for amplification of donor DNA** | |
| EcoR-Kup- F | ACAGCTATGACCATGATTACGAATTCCACGGGTGATTACTTGTTTACAT |
| Hind-Kdown-R | GTAAAACGACGGCCAGTGCCAAGCTTCAATACCGATAAAGTGGTCAACT |
| inGUT1UP-F | GTCATCCTACAGCAAACACC |
| inGUT1DO-R | TCGTGATCTTGCCGGATAAT |
| HAPTg1UP-F | CTATGACCATGATTACGAATTCGAGCT |
| HAPTg1DO-R | TGCCTGCAGGTCGACTCTAG |
| HAPAg2UP-F | GGGGTGAGCCTCAAGGTATATAG |
| HAPAg2DO-R | GGGATAGCCATCGTTTCGAAT |
| HAPFg1UP-F | ATAACTCTTGTCGATACGTATACTTGC |
| HAPFg1DO-R | ACGGACTCAACACCTTCTCC |
| **Primers for detection of gene integration** | |
| inGAPDO-F | GTCCCTATTTCAATCAATTGAACAACTAT |
| in*atX*-F | AAATGCCGGAATCGATCCCG |
| gPAOX1-DO-R-2 | CTCGGCTTGGAAGTCATCGTAA |
| in*npgA*-F | GGATTCGGCTGGGGATTTCG |
| gTEF-R | GGCAATCTCAGAGGCTTGTC |
| in*atA*-F | CTCTTTCAGCGGGTCACCCA |
| gFLD1-R | ATGTCAGCCACCACAGTGTATTG |

# Table S3 Plasmids and strains used in this study

|  |  |  |
| --- | --- | --- |
| **Plasmids** | **Characteristics** | **Source** |
| pUC18 | AmpR; *E.coli* subcloning vector | Invitrogen |
| pPIC3.5K | AmpR; HIS4; G418R; P*AOX1*-based yeast expression vector | Invitrogen |
| p414-TEF1p-Cas9-CYC1t | TRP1; p414 derivative carrying *CAS9* | Addgene [1] |
| pPIC3.5K-GUT1-gRNA1 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1* and GUT1-gRNA1 | This study |
| pPIC3.5K-GUT1-gRNA2 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1* and GUT1-gRNA2 | This study |
| pPIC3.5K-GUT1-gRNA3 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1* and GUT1-gRNA3 | This study |
| pPIC3.5K-KU70-gRNA1 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1* and KU70-gRNA1 | This study |
| pPIC3.5K-KU70-gRNA2 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1* and KU70-gRNA2 | This study |
| pUC18-DGUT1 | pUC18 derivative carrying 1000 bp upstream and downstream of *GUT1* CDS | This study |
| pUC18-DKU70 | pUC18 derivative carrying 1000 bp upstream and downstream of *KU70* CDS | This study |
| pPIC3.5K-PAOX1up-gRNA1 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1* and P*AOX1*UP-g1 | This study |
| pPIC3.5K-PAOX1up-gRNA2 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1* and P*AOX1*UP-g2 | This study |
| pPIC3.5K-PAOX1up-gRNA3 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1* and P*AOX1*UP-g3 | This study |
| pPIC3.5K-AOXTT1do-gRNA1 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1* and *AOXTT*DOWN | This study |
| pPIC3.5K-PGAP1up-gRNA1 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1* and P*GAP*UP-g1 | This study |
| pPIC3.5K-PGAP1up-gRNA2 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1* and P*GAP*UP-g2 | This study |
| pPIC3.5K-PTEF1up-gRNA1 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1* and P*TEF1*UP-g1 | This study |
| pPIC3.5K-PTEF1up-gRNA2 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1* and P*TEF1*UP-g2 | This study |
| pPIC3.5K-PFLDup-gRNA1 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1* and P*FLD1*UP-g1 | This study |
| pPIC3.5K-PFLDup-gRNA2 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1* and P*FLD1*UP-g2 | This study |
| pP-GFP | pPIC3.5K derivative carrying eGFP expression cassette | Liu et al [2]. |
| pGG | pUC18 derivative carrying eGFP expression cassette | This study |
| pDGG-PAg1 | pGG derivative carrying homologous arm of P*AOX1*UP-g1 target | This study |
| pDGG-PAg2 | pGG derivative carrying homologous arm of P*AOX1*UP-g2 target | This study |
| pDGG-PAg3 | pGG derivative carrying homologous arm of P*AOX1*UP-g3 target | This study |
| pDGG-PATg1 | pGG derivative carrying homologous arm of *AOXTT*DOWN target | This study |
| pDGG-PGg1 | pGG derivative carrying homologous arm of P*GAP*UP-g1 target | This study |
| pDGG-PGg2 | pGG derivative carrying homologous arm of P*GAP*UP-g2 target | This study |
| pDGG-PTg1 | pGG derivative carrying homologous arm of P*TEF1*UP-g1 target | This study |
| pDGG-PTg2 | pGG derivative carrying homologous arm of P*TEF1*UP-g2 target | This study |
| pDGG-PFg1 | pGG derivative carrying homologous arm of P*FLD1*UP-g1 target | This study |
| pDGG-PFg2 | pGG derivative carrying homologous arm of P*FLD1*UP-g2 target | This study |
| pBAD33-mCherry | pBAD33 derivative carrying mCherry CDS | Yang et al. [3] |
| pGAPZ-BFP | PGAPZB derivative carrying BFP CDS | Liu et al. [2] |
| 3.5k-PAg2+PTg1 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1*, P*AOX1*UP-g2and P*TEF1*UP-g1 | This study |
| 3.5k- PFg1+PTg1 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1*, P*FLD1*UP-g1and P*TEF1*UP-g1 | This study |
| 3.5k-PFg1+PAg2+PTg1 | pPIC3.5K derivative carrying PARS, *CAS9*, P*HXT1*, P*FLD1*UP-g1, P*AOX1*UP-g2and P*TEF1*UP-g1 | This study |
| pDGChy-PTg1 | pUC18 derivative carrying mCherry expression cassette and homologous arm of P*TEF1*UP-g1 target | This study |
| pDGChy-PFg1 | pUC18 derivative carrying mCherry expression cassette and homologous arm of P*FLD1*UP-g1 target | This study |
| pDGChy-PAg2 | pUC18 derivative carrying mCherry expression cassette and homologous arm of P*AOX1*UP-g2 target | This study |
| pDGB-PTg1 | pUC18 derivative carrying BFP expression cassette and homologous arm of P*TEF1*UP-g1 target | This study |
| pDGB-PAg2 | pUC18 derivative carrying BFP expression cassette and homologous arm of P*AOX1*UP-g2 target | This study |
| pDGB-PFg1 | pUC18 derivative carrying BFP expression cassette and homologous arm of P*FLD1*UP-g1 target | This study |
| pPICZB-atA | pPICZB derivative carrying *atA* gene | Kong et al.  [4] |
| pPIC3.5K-pGAP-npgA | pPIC3.5K derivative carrying *npgA* gene | Gao et al. [5] |
| pPIC3.5K-pGAP-atX | pPIC3.5K derivative carrying *atX* gene | Gao et al. [5] |
| pDTg1 | pUC18 derivative carrying homologous arm of P*TEF1*UP-g1 target | This study |
| pDAg2 | pUC18 derivative carrying homologous arm of P*AOX1*UP-g2 target | This study |
| pDFg1 | pUC18 derivative carrying homologous arm of P*FLD1*UP-g1 target | This study |
| pDTg1-npgA | pDTg1 derivative carrying *npgA* gene | This study |
| pDAg2-atX | pDAg2 derivative carrying *atX* gene | This study |
| pDFg1-atA | pDFg1 derivative carrying *atA* gene | This study |
| ***P. pastoris*** | **Characteristics** | **Source** |
| GS115 | *his4*, *AOX1*, *AOX2* | Invitrogen |
| Δ*ku70* | *his4*; GS115 *KU70*Δ | This study |
| K-NX | Δ*ku70* with *atX* and *npgA* gene | This study |
| K-NXA | Δ*ku70* with *atX*, *npgA* and *atA* gene | This study |
| ***E. coli*** | **Characteristics** | **Source** |
| TOP10 | F’ [lacIq, Tn10(TetR)] *mcr*A φ80 *lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 | Invitrogen |

# Table S4 Variable gRNA binding sites used in gene deletion

|  |  |
| --- | --- |
| **Name** | **Sequence (5’→3’)** |
| GUT1-gRNA1 | CGAGTACTCTACCTCTGCTC |
| GUT1-gRNA2 | TGCAATTTCCTCAGCCAGGC |
| GUT1-gRNA3 | GTTGTTTGGTCCAAGAAGAC |
| KU70-gRNA1 | CATCTTAGAGAATGTCAGTG |
| KU70-gRNA2 | CCTTGCCCGGTACAGGAATA |

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